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Genetic and Biochemical Characterization of Waxy Mutants in Cereals

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During fine structure analyses of artificially induced waxy mutants in maize by use of Nelson's pollen analysis methods, it was noticed that some mutants showed a phenotype intermediate between waxy and normal nonwaxy. Such intermediate or leaky waxy mutants were frequent among mutants induced by a chemical mutagen, EMS. They seemed to be located evenly within the waxy locus. This would suggest that they might have been missense mutations, which would produce full-sized but partially inactivated enzymes, rather than deletions or frameshift mutations. To confirm the intermediate phenotype, a rapid measuring system was developed to measure waxyness of endosperm quantitatively. It was based on the blue value method and is applicable to a single grain of rice. Among the 27 waxy mutant lines of maize, including 11 EMS-induced, two of the EMS-induced mutants were clearly intermediate. Eighteen EMS induced *wx* mutants of rice were also examined, and nine were intermediate.

Introduction

Pollens of higher plants have many advantages in genetical analyses. They are haploid germ cells and can be examined in very large population if appropriate genetic trait is used. Fine structure analyses of waxy (*wx*)(*1-3*) and amylose extender (*ae*) (*4*) in maize as well as glutinous (*gl*: comparable to *wx*) in rice (*5*) are examples of successful analyses utilizing the merits of pollen analysis. Intralocus mapping of mutant alleles would be a good means to see possibilities of the presence of point mutations in higher plants. To obtain materials for such analyses, many *wx* mutants have been induced in maize by using ionizing radiations, germicidal ultraviolet light, or chemical mutagens (*6-8*). In these experiments, *wx* mutants were detected after a test cross of the treated materials with a standard tester *wx* stock. In the case of ultraviolet light, mutants were detected after self-pollination, but later they were crossed to the tester *wx* to confirm allelism. Therefore all of these waxy mutants belonged to the *wx* locus on chromosome 9. During fine structure analyses of these *wx* mutants by using Nelson's pollen analysis method (*2*), it was noticed that some mutants showed intermediate phenotype between *wx* and normal nonwaxy (*Wx*) (*9*). An example of such an intermediate *wx* mutant is shown in Figure 1. Here the intermediate *wx* mutant had been crossed to one of the standard *wx* mutant (wx^{90}), and segregating pollen were stained with iodine. The dark pollen at the center is considered to be a nonwaxy recombinant which may serve as *Wx* color standard in the same preparation. Other pollen grains segregated into light-colored *wx* and intermediate-colored mutant pollen in a theoretical one to one ratio. Those intermediate *wx* mutants were stable in their degree of waxyness. Such intermediate or leaky waxy phenotypes were inherent in some of the mutants induced by a chemical mutagen, ethyl methanesulfonate (EMS). They seemed to be located evenly within the waxy locus (*9*). They might have been missense mutations which might produce protein or enzyme with normal length but its function partially inactivated. To confirm intermediate phenotypes, a rapid measuring system was developed to measure waxyness of

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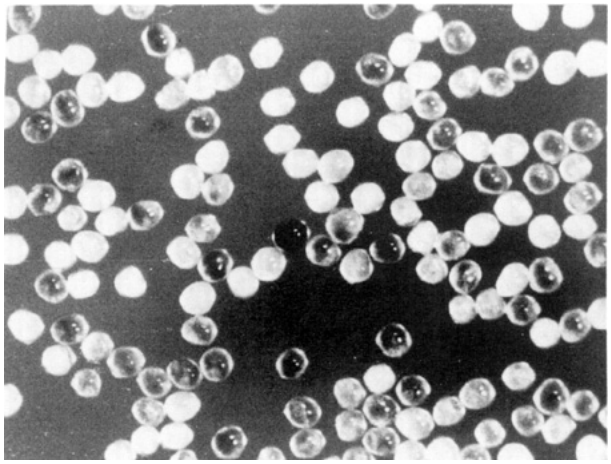


FIGURE 1. Pollen grains of maize segregating standard *wx*^{m} and intermediate *wx* after iodine stain. Very dark pollen grain at the center is considered to be nonwaxy recombinant.

endosperm starch quantitatively. It was based on the blue value method to measure amylose fraction of starch (*10*). Results of measurements of *wx* mutant lines in maize and also in rice are reported here. Distribution characteristics of the waxyness indices were also discussed.

Materials and Methods

Plant Materials

Normal nonwaxy (*Wx*) strain of maize (6311R) used was a multiple dominant genetic stock with purple plant color. Standard waxy (*wx*) mutant strain of maize (639) was a multiple recessive genetic stock with *C* sh_1 and *bz* marker genes. These stocks were originally obtained from Dr. H. H. Smith, Brookhaven National Laboratory. Other genetic stocks were kindly supplied by Dr. R. W. Briggs (Funk Seeds International), Dr. O. E. Nelson (University of Wisconsin), and Maize Genetics Cooperative. Other waxy mutants were induced in the strain 6311R by seed treatment with radiations or EMS, or pollen irradiation by germicidal ultraviolet light. Most of the materials were used with 6311R background.

The standard waxy starch mutant of rice, 504T65wx, was obtained from Dr. H. Morishima (National Institute of Genetics). Other *wx* mutants in rice examined were induced by EMS treatment of seeds of a normal nonwaxy Japonica variety, Norin No. 8 (11, 12). These materials were self-pollinated and selected for normal growth for a few generations.

Preparation of Starch Solution

Measurements were done on a kernel basis. Each whole maize kernel was crushed to coarse meal. The kernel was placed in a plastic cylinder on a piece of iron plate. A short iron rod was inserted into the cylinder and was hit with a hammer. The meal was transferred to a test tube. A 5-ml portion of distilled water was added to each tube. For rice, hulls were removed, and each grain was crushed with pliers and put into a test tube. A 3-ml portion of water per tube was used for rice. The test tubes were autoclaved at 1 atm for 15 min. After stirring for several seconds while hot, they were left overnight for cooling and sedimentation of debris. The supernatant fluid was used in the measurement.

Iodine Reagent and Staining

Half strength of Nelson's I_2–KI solution (*2*) was used to stain the starch solution. The recipe adopted was 450 mg iodine and 2.5 g KI in 500 ml of water. To reduce the effect of color of excess iodine reagent, staining was done in excess of starch. A 1-ml portion of sample starch solution was mixed with 0.1 ml of iodine reagent and 2 ml of water in a 10 mm × 10 mm glass absorption cell. The cell was immediately set in the colorimeter. The measurement was completed within 1 min.

Colorimetry

The colorimetric apparatus was designed to measure with two wavelengths (430 nm and 660 nm) simultaneously (*13,14*). As shown in Figure 2, it

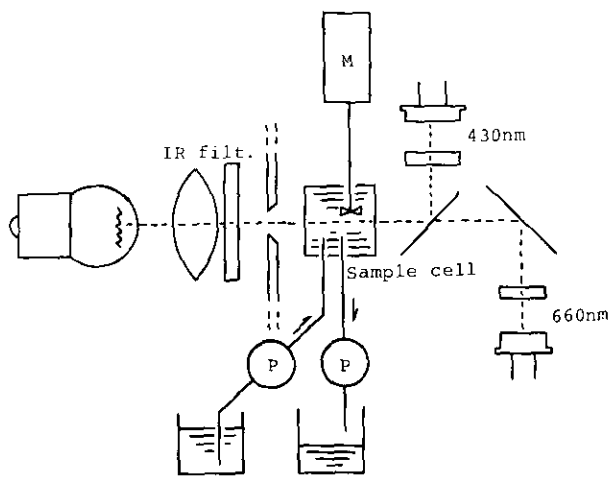


FIGURE 2. Schematic of colorimeter. Absorption cell is equipped with inlet, outlet tubes and a stirring blade for dilution. Light beam was divided by mirrors and measured by silicon photocells behind interference filters.

consisted of an incandescent lamp, an absorption cell, mirrors to divide the light beams, interference filters, and silicon photocells of photometry grade (Hamamatsu TV, S780-8BK). A small mixing blade and inlet and outlet capillaries were inserted into absorption cell so that continuous dilution could be done while concentration was monitored photometrically. When the sample solution was diluted to 50% transmittance for 430 nm, an electric signal was produced to command a digital printer to print out the transmittance value for 660 nm. This transmittance value for 660 nm was used as index to express waxy phenotype. Monochromatic lights obtained by using interference filters were used for high reproducibility.

Results

The rationale of the photometry at two wavelengths is to measure amylose which is deleted in the *wx* phenotype, by the blue value method at a certain concentration of starch solution which was determined photometrically. Amylopectin is a major and common fraction in both types of starches. In *Wx* starch, amylopectin amounted to about 75% and in *wx* starch it reached almost 100% (*15-17*). In solution, amylopectin is stained reddish purple by iodine (*18*). To bring the concentration of sample solution to a predetermined level, the iodine-stained solution was diluted continuously while monitoring the concentration of iodine-amylopectin complex by 430 nm blue light. When the concentration reached to 50% transmission at 430 nm, the percent transmission (*%T*) value at 660 nm red light was measured and recorded by a digital printer. This *%T* value at 660 nm represents the concentration of the blue colored amylose-iodine complex.

For preparation of the starch solution, alkaline dispersion was also tested. Starch could be dispersed well from the crushed endosperm by 1*N* KOH solution. However, it was not used in the present measurements for two reasons: first, it requires neutralization of each sample solution before iodine staining; second, it reduced the difference of indices between *Wx* and *wx*. Compared to alkaline dispersion, autoclaving was convenient and good for uniform preparation of many sample solutions. The time of autoclaving was varied, and no significant difference was found from 5 to 30 min. A period of 15 min was adopted because it is the same condition as routine sterilization procedure in our laboratory. Measurements of waxy phenotype were made for each kernel. Frequency distribution of the indices for waxy phenotype were examined. The mean value of the indices of *wx* lines in maize ranged from 63 to 70. In rice, the values were a little higher both in *Wx* and *wx* lines. Figures 3 and 5 show distribution of *wx* indices of each kernel of the *wx* mutants of maize and rice respectively. High reproducibility of the measurements is demonstrated in strain 6311R (*Wx*) in Figure 3. M14, another *Wx* inbred line, showed wider distribution of the waxy indices probably inherent to this line. In Figures 4 and 6, *wx* mutants were placed in orders of mean *wx* indices of each *wx* mutant line.

In maize, most of the 27 *wx* mutant lines examined were complete *wx* mutants (Fig. 4). Eight *wx* mutant lines were genetic stocks of translocation or other phenotypes. Those *wx* genes might be presumably of the same origin. 1J2, R, A39, 1M2, 90

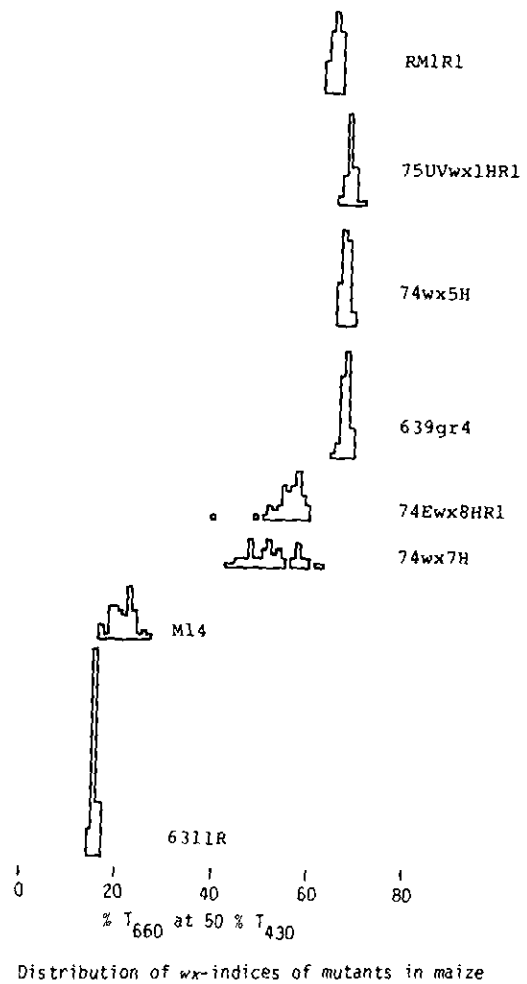


FIGURE 3. Distribution of waxyness indices of kernels of *wx* mutants of maize. Each starch solution was prepared from mature endosperm by autoclaving. Iodine stained solution was diluted while measuring. The transmission value of 660 nm light at the concentration to give 50% transmission of 430 nm light was used as index to express waxyness.

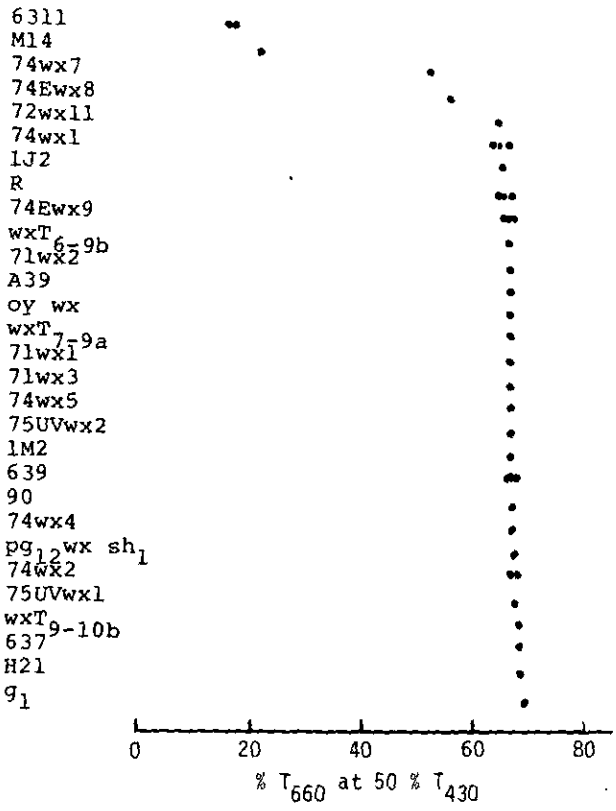
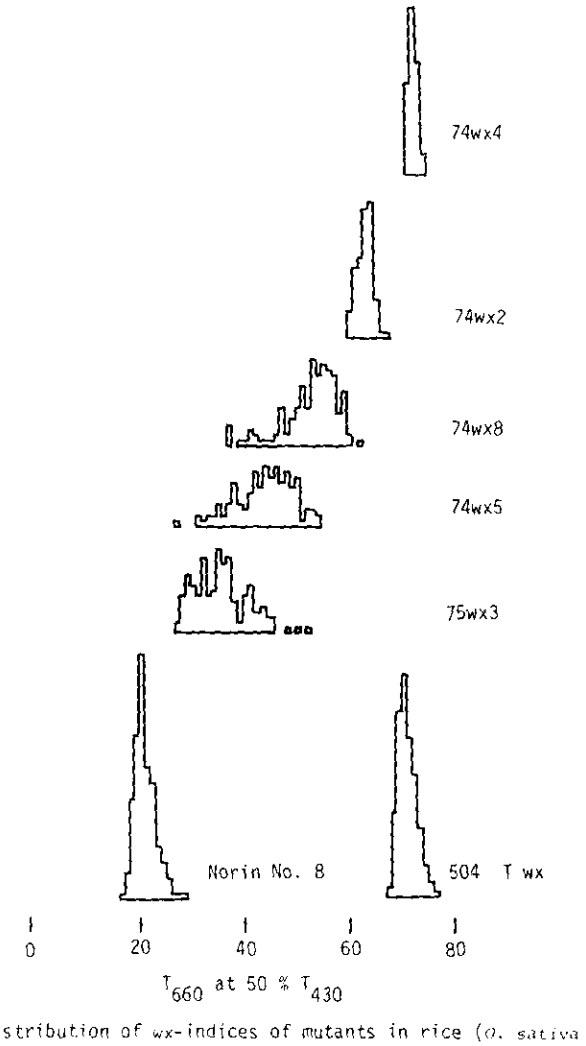


FIGURE 4. Waxyness indices of *wx* mutants of maize. 6311 and M14 are normal *Wx* imbred lines. Mutants with prefix figures of 71, 72, and 74 are EMS-induced, and of 75UV are ultra-violet light-induced waxy mutants. Each point represents mean value of a single plant.



Distribution of wx-indices of mutants in rice (*O. sativa*

FIGURE 5. Distribution of waxyness indices of kernels of *wx* mutants of rice (*O. sativa* var. Norin No. 8). Norin No. 8 is a normal *Wx* line. Other *wx* mutants were induced by EMS.

and H21 are known to be mutants of independent origins. 75UVwx1 and 75UVwx2 were mutants induced by irradiation of pollen grains with germicidal ultraviolet light. Mutants with prefixes 71, 72, and 74 were EMS-induced mutants. Of 11 EMS-induced *wx* mutants, two were clearly intermediate (Fig. 3) and another two were next to them (Fig. 4).

In rice, some *wx* mutants were very close to normal *Wx* (Figs. 5 and 6). The mutant 74wx3 had been found as doubtful *wx* mutants. Examination of waxy indices on a kernel basis revealed that the distribution of the indices was extended to higher region compared to normal *Wx*, indicating that amylose content was affected to some extent, though the mean value of the indices did not differ significantly. Here, the line 74wx3 was considered very leaky *wx* mutant. Among the 18 EMS-induced *wx* mutant lines, nine could be classified as intermediate *wx* mutants.

Compared to maize, in which *wx* mutants were detected after a test cross with a standard *wx* line, *wx* mutant in rice were detected in M_2 endosperms on self-pollinated M_1 panicles. Accordingly allelism tests would be necessary for further examination of proportion of the intermediate *wx* mutants in rice. In the present experiment, the means of indices of intermediate *wx* mutant lines were distributed continuously from *Wx* to *wx*.

Discussion

The nature of mutation in higher plants has been studied extensively by many investigators in related fields. After the suggestion by Stadler and Roman (*19*) that most radiation-induced mutations in plants might be due to deletion of a small chromosome segment, extra care was taken in use of the term, point mutation or gene mutation.

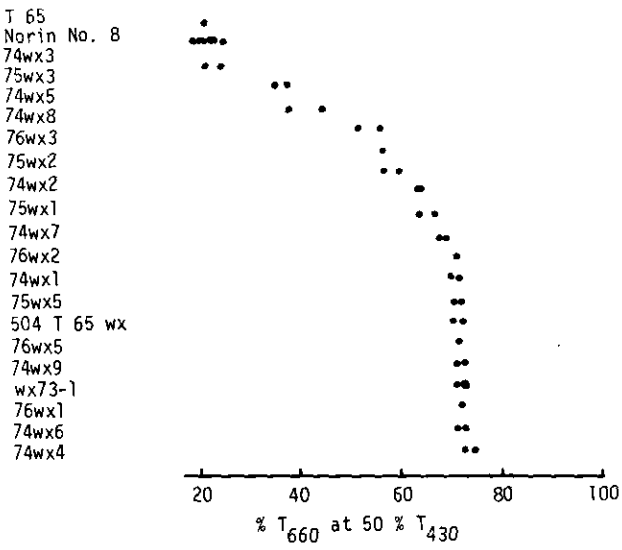


FIGURE 6. Waxyness indices of *wx* mutants of rice. T65 and Norin No. 8 are normal *Wx* lines. 504T65 wx is a standard *wx* marker line with T65 back ground. Others are EMS-induced *wx* mutants. Each point represents mean value of a single plant.

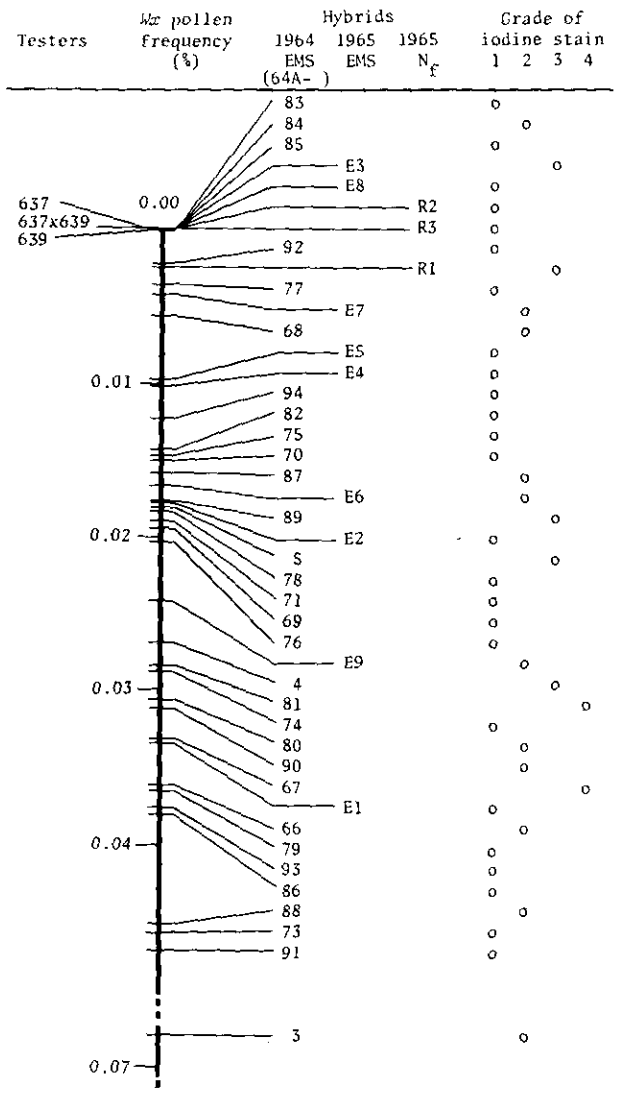


FIGURE 7. Distribution of induced *wx* mutants as placed by *Wx* pollen grain frequencies in hybrids between tester *wx* and the mutants. Arbitrary grading of iodine stain was made between (1) no background segregation, and (4) half of the *wx* pollen stained so dark that scoring of *Wx* needed extra care in staining [from Amano (*9*)].

However, induction of a point mutation in its strict definition, single base-pair substitution, and its confirmation became possible by the recent developments of studies on chemical mutagenesis (*20-22*). Detailed analyses of mutant characters and knowledge of biochemistry concerning both in chemical reactions in mutagenesis (*23-25*) and gene function (*26*) would support this view. Intralocus fine structure analysis developed by Nelson (*1*) would have limited resolution in measuring the size of genetic alteration which caused the mutation. Immunological detection of CRM and electrophoretic detection of modified protein might be a good means to show that the mutation concerned was a missense type base pair substitution. These methods were successfully applied to sh_1 locus mutants in maize (*27*). CRM and modified proteins were detected in some EMS-induced sh_1 mutants. Intermediate or leaky phenotypes reported here would be another examples to indicate, though indirectly, the presence of full size protein molecules which is affected its function only partially. The intermediate phenotypes reported here in maize were seemed to be inherent to certain EMS induced *wx* mutant lines. These *wx* mutant genes were allelic to other *wx* mutants suggesting that they were in the same *wx* cistron.

A modifier of *Wx-wx* phenotype, amylose extender (*ae*), is known on a different chromosome. It is a recessive gene and increases the amylose or amyloselike fraction in starch both in *Wx or wx* phenotypes (*15, 16, 28*). But the present examples of the intermediate *wx* mutant line are not of this case. There was no phenotypic expression of *ae-wx* double mutations (*15*), and no *ae-Ae* segregation was observed. It can be also noticed from Figure 7 that there are too many intermediate *wx* mutants induced by EMS to permit them to be ascribed to simultaneous mutation at *ae* locus. In Figure 7, besides the recombinational distances from the standard *wx* stock, 639, expressions of *wx* phenotype as observed after iodine staining of pollen grains were classified into four groups. The

classifications were made between colors of standard *wx* and normal *Wx* recombinant pollen grains in the same slide preparation. Of the 40 EMS-induced *wx* mutants in the same *wx* cistron, 17 mutants (42.5%) were more or less intermediate. It would be very likely that these intermediate *wx* phenotypes were inherent in some of the induced *wx* mutant genes due to base-pair substitution of missense type. Unfortunately, the *wx* mutants in Figures 4 and 7 were not able to be compared, because of the experimental difficulty in establishing and confirming the newly induced *wx* mutant line in maize in the early phase of the studies. Presently, a lower proportion of intermediate *wx* mutant among EMS-induced *wx* mutants in maize, compared to 42.5% occurrence in pollen analyses, was obtained by analyses of endosperm starch. But the number of *wx* mutant lines analyzed might be too small to permit generalization of the results. Actually in rice experiments, a much higher proportion of intermediate *wx* mutant lines could be induced by EMS, and this was shown by endosperm analyses. In rice, *wx* mutants were detected in M_2 endosperms on M_1 panicles after self-pollination. They were easily established as homozygous mutant lines. Although allelism tests must be completed for further discussions on location and other basic characteristics of the mutant genes, 9 out of 18 EMS mutant induced *wx* mutants were considered to be intermediate *wx* mutant lines as shown in Figure 6. Mean *wx* indices for each *wx* mutant line were distributed rather continuously from the *Wx* region (74wx3)to fully *wx* region (74wx7). Since in these analyses all viable *wx* mutants were analyzed, it might be comparable to the results shown in Figure 7, as for sampling of materials. In both materials, EMS treatments were made under conditions favorable for less chromosome aberration (*29*). Simply, EMS was dissolved in distilled, deionized water without use of any buffering salts. The solution was acidic and the pH was around 3. High efficiency in induction of mutation and good survival of the induced mutant (*7, 9*) would support the explanation of intermediate *wx* phenotype by missense mutation. Intermediate phenotypes were also observed in some of the non-C^I (*9*) or sh_1 mutations. These facts would suggest that an intermediate phenotype might be a rather common phenomenon where point mutations could occur.

In conclusion, it is advised that the monitoring system for mutagenic activity of environmental pollutants be prepared also for intermediate mutant phenotypes if point mutation inducers are expected.

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